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Regulation of Membrane Trafficking: Structural Insights from a Rab/Effector Complex

Minireview

Lino Gonzalez, Jr. and Richard H. Scheller*
Howard Hughes Medical Institute
Department of Molecular and Cellular Physiology
Stanford University
School of Medicine
Stanford, California 94305

Introduction

Eukaryotic cells contain a highly dynamic set of membrane compartments that are responsible for packaging, sorting, secreting, and recycling proteins and other molecules. Trafficking between organelles within the secretory pathway occurs as vesicles derived from a donor compartment fuse with specific acceptor membranes, resulting in the directional transfer of cargo molecules. This process is tightly controlled by the Rab/Ypt family of proteins (reviewed by Novick and Zerial, 1997), a branch of the superfamily of small GTPases. Rab proteins regulate a variety of functions, including vesicle translocation and docking at specific fusion sites. Rabs may also play critical roles in higher order processes such as modulating the levels of neurotransmitter release in neurons, a likely mechanism in synaptic plasticity that underlies learning and memory (Geppert and Südhof, 1998).

Small GTPases share a common three-dimensional fold that, in the GTP bound state, can bind a variety of downstream effector proteins. GTP hydrolysis leads to a conformational change in the "switch" regions that renders the GTPase unrecognizable to its effectors. In this way, by localizing and activating a select set of effectors, a common structural motif is used to control a wide array of distinct cellular processes. Recently, the first structure of a Rab/effector complex was reported for the Rab-binding domain (RBD) of the Rab effector protein Rabphilin-3A bound to Rab3A (Ostermeier and Brünger, 1999). This structure highlights a novel RBD and sheds light on the specificity of effector interactions within the Rab family, interactions that may be critical for understanding the specificity and regulation of membrane trafficking in cells.

The Role of Rabs in Regulating Membrane Trafficking

The final steps in membrane fusion are likely to be driven by a set of proteins known as SNAREs (Figure 1). After a vesicle becomes docked, the cytoplasmic domains of VAMP (also termed synaptobrevin) and syntaxin on opposing membranes, in combination with a SNAP-25 molecule, coalesce into an elongated α-helical bundle (Poirier et al., 1998; Sutton et al., 1998), which may lead to fusion. Because numerous SNARE isoforms have been identified that localize to distinct membrane compartments, it was originally proposed that the specificity of interaction between the SNARE proteins accounted for the specificity in membrane trafficking. Recent results, however, suggest that SNAREs are not specific in their ability to form complexes in vitro, suggesting that trafficking specificity requires additional factors (Yang et al., 1999). In this regard, Rab proteins are strong candidates for governing the specificity of vesicle trafficking. Like the SNAREs, many isoforms ($\sim$40) of the Rab family have been identified that localize to specific membrane compartments (reviewed by Novick and Zerial, 1997).

Concomitant with the SNARE cycle, Rab proteins undergo a intricate cycle of membrane and protein interactions. Rabs are posttranslationally modified at C-terminal cysteines by the addition of two geranylgeranyl groups, which mediate membrane association when the Rab is in the GTP-bound state (Figure 1). After guanine nucleotide hydrolysis occurs, the Rab is extracted from the membrane upon forming a complex with a cytosolic GDP-dissociation inhibitor (GDI). This cytosolic intermediate is then recycled onto a newly forming vesicle, most likely through a secondary factor termed a GDI dissociation factor (GDF), which displaces GDI. After the Rab becomes membrane bound, a guanidine nucleotide exchange factor (GEF) promotes release of GDP and the subsequent loading of GTP. In its GTP-bound conformation, the Rab is then free to associate with its specific set of effectors, which can in turn trigger events leading to the eventual fusion of the vesicle with a target membrane. To complete the cycle, perhaps after or concurrent with membrane fusion, a GTPase activating protein (GAP) accelerates nucleotide hydrolysis, switching off the GTPase. The remaining GDP-bound Rab can then participate in a new round of fusion.

Rab interactions with effectors are likely to regulate vesicle targeting and membrane fusion in three ways (Figure 1). First, a Rab may specifically facilitate vectorial vesicle transport. Vesicles are transported from their site of origin to acceptor compartments likely through associations with cytoskeletal elements and transport motors. A protein has been identified with a domain structure that suggests a connection between the cytoskeleton and the Rabs. This protein, called Rabkinesin-6, contains a kinesin-like ATPase motor domain followed by a coiled-coil stalk region and a RBD that specifically binds Rab6 (Echard et al., 1998). An additional link with the cytoskeleton is provided by the Rab effector, Rabphilin-3A. Rabphilin-3A has been shown in vitro to interact with α-actinin, an actin-bundling protein, but only when not bound to Rab3A (Kato et al., 1996). These results raise the intriguing possibility that Rab proteins regulate vesicle interactions with the cytoskeleton and thereby play an active role in targeting vesicles to their appropriate destinations.

Second, Rab proteins may regulate membrane trafficking at the vesicle docking step. A number of Rab effectors, including Rabaptin-5, EEA1, Rabphilin-3A, and Rim, may serve as molecular tethers. Each effector protein contains a RBD, followed by a linker region (some having the potential to form elongated coiled-coil structures), and a domain capable of interacting with a

*To whom correspondence should be addressed (e-mail: scheller@cmgm.stanford.edu).

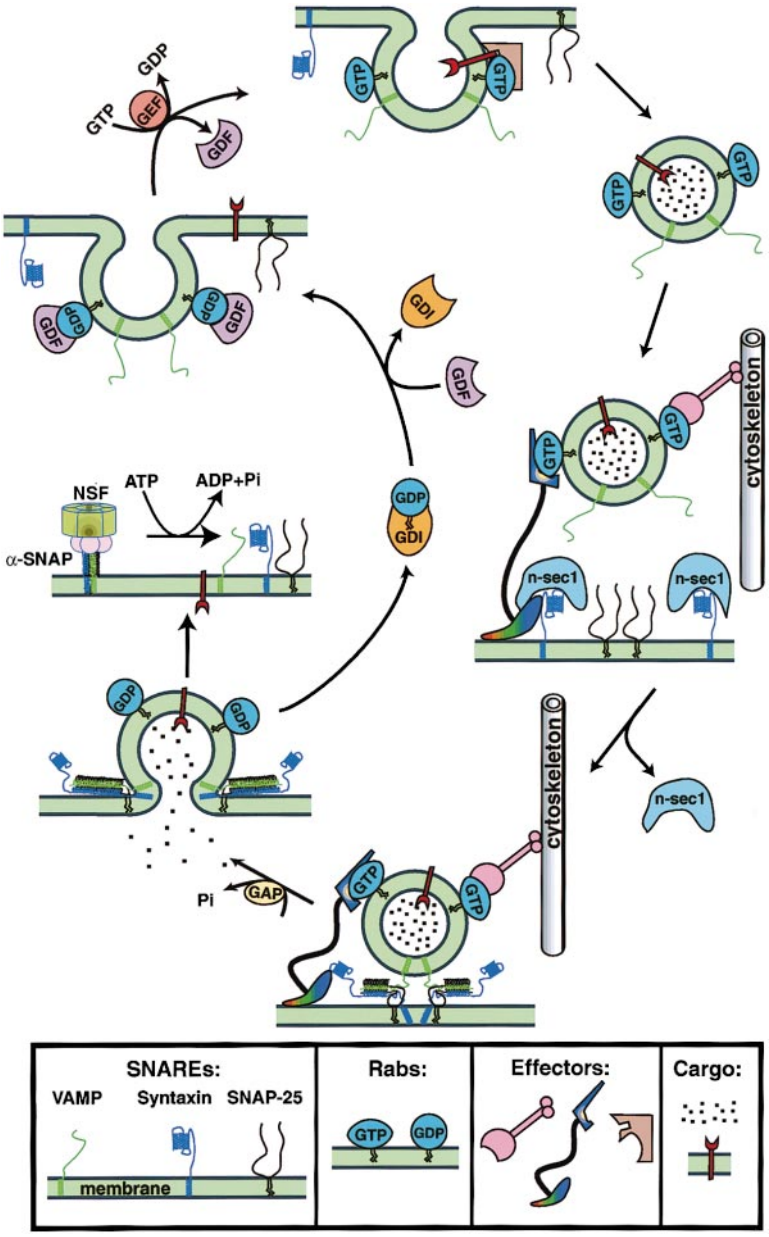

Figure 1. Composite Model of the Rab and SNARE Functional Cycles

A description of the Rab cycle is included in the text. Once recruited to a newly formed vesicle and activated by binding GTP, a Rab protein can interact with a select set of effectors (illustrated as generic effectors) and implement various activities in the membrane trafficking process. For example, an effector, such as TIP47 (Díaz and Pfeffer, 1998), might facilitate cargo selection by linking specific cargo molecules to a specific Rab protein (top panel, brown effector). Next, Rabs may function through effector proteins that translocate and dock the vesicle to a target membrane (central right panel). Once docked, Rab effectors may activate the fusion machinery through interactions with SNARE regulatory proteins, such as n-sec1 (also termed munc-18), freeing SNAREs to associate into elongated α-helical bundles, leading to membrane fusion (bottom and lower left panels). At each of these steps, Rab proteins may impart specificity to the overall process of membrane trafficking. Following fusion, the SNARE complex is disassembled into individual components through the actions of the ATPase NSF and α-SNAP (central left panel). For vesicle SNAREs to function in an additional cycle, they must be returned to a donor membrane via retrograde transport.

second Rab or the target membrane. Rabaptin-5, for example, contains two RBDs, one near the N terminus that specifically recognizes Rab4 and a second near the C terminus that binds Rab5 (Vitale et al., 1998). Both Rim, which is localized to the target membrane, and Rabphilin-3A, which is localized to the vesicle, contain N-terminal RBDs and C-terminal Ca^{2+}-binding C2 domains, implicating these effectors in synaptic vesicle localization or docking in response to Ca^{2+} influx (Wang et al., 1997). Tethering effectors may also recognize protein complexes on the acceptor membrane. Sec4p, a yeast Rab3A homolog, interacts with the exocyst (Guo et al., 1999), a complex of seven or more subunits that is assembled at sites of vesicle fusion along the plasma membrane. The exocyst complex may therefore function as a landmark for Rab/effector-mediated vesicle docking.

Third, once a vesicle has become tethered to its fusion site, Rab proteins may selectively activate the SNARE fusion machinery. The mechanism of this activation is unknown but may involve direct interactions of Rabs or, more likely, their effectors with SNAREs. For example, Hrs-2 is a protein that binds to SNAP-25 and contains a Zn^{2+}-finger motif characteristic of Rab-binding proteins such as Rabphilin-3A, Rim, EEA1, and Noc2, suggesting that Hrs-2 may form a physical link between Rabs and SNAREs (Bean et al., 1997). In addition, certain mutations in the syntaxin-binding protein Sly1p, the Sec1p homolog utilized in ER to Golgi trafficking, eliminate the requirement for Ypt1p, a Rab protein that functions at this trafficking step (Dascher et al., 1991). Rabs may therefore regulate SNARE associations through Sec1 family members (Figure 1). In support of this idea, a Rab effector was recently found to interact with a vacuole

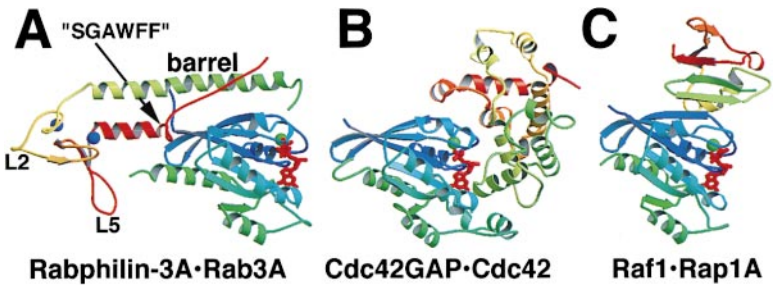

Figure 2. The Diversity of Protein Binding Surfaces on Small GTPases

Crystal structures of the small GTPases Rab3A (A), Cdc42 (B), and Rap1A (C) are colored dark blue to green from N to C termini, respectively. The three GTPase domains have been similarly oriented. For spatial reference, the guanine nucleotide is colored red, and the Mg^{2+} ions are represented by green spheres. Each of the small GTPase-binding proteins are colored green to red from N to C termini, respectively.

(A) Rabphilin-3A binds Rab3A at the "switch" regions located on the top side of the GTPase, near the Mg^{2+} ion, and in a pocket located on the side opposite the nucleotide. The Zn^{2+} ions, represented as blue spheres, are located in the Zn^{2+}-binding subdomain on the left. Loop regions 2 and 5 are labeled L2 and L5, respectively.

(B) The Cdc42GAP protein binds to Cdc42 on the nucleotide binding side of the GTPase and contacts the "switch" regions.

(C) The effector domain of Raf1 binds to Rap1A mainly through a β sheet interaction near the "switch" region. Coordinates for the Rabphilin-3A·Rab3A complex were kindly provided by Dr. Axel Brunger. Other coordinates were obtained from the Protein Data Bank under the accession codes: 1GRN for Cdc42GAP·Cdc42; 1GUA for Raf1·Rap1A. This figure was created using MOLSRIPT and Raster3D (see references in Nassar et al., 1998).

Rab, a Sec1p homolog, and a SNARE protein (Peterson et al., 1999), which suggests that this effector serves to connect Rab and SNARE function. In this way, Rabs and their effectors may facilitate the correct pairing of SNAREs.

Structural Basis of Rab/Effector Interactions

Rab3A is the most abundant Rab protein localized to synaptic vesicles. Genetic studies in *C. elegans* and mice reveal only mild behavioral deficits in Rab3 mutant organisms, consistent with a nonessential regulatory role for this protein in the synaptic vesicle life cycle. In *C. elegans*, Rab3 null mutants have only 40% of the normal levels of vesicles at active zones and more vesicles are found along the axon at nonsynaptic regions. These data suggest that Rab3 functions in the recruitment or sequestration of vesicles to active zones (Nonet et al., 1997). In Rab3A knockout mice, the morphology of the synapse is normal. However, physiological analysis of hippocampal synapses supports a role in either recruitment of vesicles to the active zone or, more likely, regulation of the fusion process itself (Geppert and Sudhof, 1998 and references therein). The precise mechanism of Rab3A function in the nervous system remains unclear; however, interactions with effector proteins such as Rabphilin-3A are likely to be important.

From the preceding, it is clear that knowledge of the structural basis by which Rab proteins selectively interact with their effectors will be critical for understanding Rab function. Ostermeier and Brunger have taken the first step in this direction by determining the 2.6 Å X-ray crystal structure of the Rabphilin-3A RBD bound to Rab3A. The RBD of Rabphilin-3A spans approximately 120 amino acids and resembles a pistol (Figure 2A). The barrel of the pistol is composed of an extended, 34–amino acid α helix, which is followed by a subdomain consisting of two Zn^{2+}-binding sites and several interspersed loops, one resembling the pistol's handle. From this loop, a second, shorter α helix runs antiparallel to the barrel and ends abruptly in a turn composed of a conserved "SGAWFF" motif, representing the pistol's trigger. Finally, along one side of the barrel α helix, the remaining 12 residues adopt an extended conformation.

The Zn^{2+}-binding subdomain of Rabphilin-3A is far removed from the interactions with Rab3A. Interestingly, however, mutations that disrupt Zn^{2+} binding abolish Rab binding, suggesting that the Zn^{2+}-binding subdomain serves as a structural scaffold. In addition, two extended loop regions, loop2 and loop5 (the handle), may serve as binding sites for downstream factors, as suggested by the C-terminal Zn^{2+}-binding domain of EEA1, which can interact with phosphotidylinositol-3-phosphate (Simonsen et al., 1998).

The GTP-bound Rab3A structure is similar to the consensus small GTPase fold, with a central six-stranded β sheet surrounded by α helices, forming a pocket at one end that cradles the GTP·Mg^{2+} (Figure 2A). Two "switch" regions are in proximity of the γ-phosphate of GTP, rendering their conformation sensitive to GTP hydrolysis. The 2.0 Å crystal structure of the uncomplexed Rab3A reveals that the structure is largely unchanged in the absence of Rabphilin-3A (Dumas et al., 1999).

Rab3A contacts Rabphilin-3A on three surfaces: (1) under the first half of the barrel α helix, (2) under the C-terminal extended region, and (3) at the "SGAWFF" trigger region (Figure 2A). The barrel α helix binds between the two conformationally sensitive "switch" regions on Rab3A, whereas the extended region contacts only one of the "switch" regions. The "SGAWFF" motif fits into a pocket located on the side opposite the nucleotide binding site.

Interestingly, the barrel α helix of Rabphilin-3A may sterically preclude GAP binding in the Rab3A "switch" regions, as suggested by the structure of the small GTPase, Cdc42, bound to its GAP (Figure 2B) (Nassar et al., 1998). In this way, Rabphilin-3A may stabilize its own association with Rab3A by blocking GAP activity. Indeed, in vitro, Rabphilin-3A strongly inhibits GTPase stimulation of Rab3A by Rab3A-GAP (Kishida et al., 1993).

Upon binding, Rab3A and Rabphilin-3A bury a substantial amount of surface area, comparable to the area buried in other stable protein complexes. Most of the side chain interactions between Rab3A and Rabphilin-3A are hydrophobic in nature. Moreover, the few salt bridge interactions that are present are not strictly conserved between Rabphilin-3A and Rim, both of which specifically bind Rab3A. Thus, complementarity of hydrophobic side chain packing must be an important

source of specificity. A comparison of the Rabphilin-3A and Rim sequences suggests that the effector residues on the barrel α helix that contact the Rab "switch" regions are not strictly conserved. This lack of sequence conservation suggests that the barrel α helix may impart only limited binding specificity and instead function primarily as a Rab conformation sensor.

Three complementarity-determining regions (CDRs) on the Rab are proposed by Ostermeier and Brunger to be responsible for selective effector interactions. These three regions make up the "SGAWFF"-binding pocket. In contrast to the "switch" regions, Rab CDRs exhibit a high degree of sequence variability. This observation, combined with the conservation of the "SGAWFF" motif in effectors that bind Rab3A, suggests that the CDR pocket provides the necessary binding specificity. Interestingly though, the C-terminal Zn^{2+}-binding domain of the Rab5 effector EEA1 ends just before the "SGAWFF" motif. Therefore, the Zn^{2+}-binding domain of EEA1 may not bind Rab5 in a manner analogous to the interaction between Rab3A and Rabphilin-3A. A recent study has shown that a 70–amino acid sequence in EEA1 immediately N-terminal of the Zn^{2+}-binding domain constitutes the minimal Rab5-binding region (Simonsen et al., 1998). This region shares homology with the Rab5-binding region of another Rab effector, Rabaptin-5, which lacks the Zn^{2+}-binding domain altogether. It is possible that the RBD of EEA1 adopts an altered topology, with respect to Rabphilin-3A, where the Zn^{2+}-binding subdomain is situated at the C terminus rather than in the middle. To gain further insight into the selectivity of Rab/effector interactions, it will be important to determine if this 70–amino acid Rab5-binding domain is structurally related to Rabphilin-3A or constitutes a novel class of RBDs.

As new Rab effectors are identified, different classes of RBDs will likely emerge. Rabkinesin-6, for example, reveals no detectable Zn^{2+}-binding domain. In addition, p40, a Rab9 effector, contains six internal repeated sequences of ~50 amino acids that are predicted to form four-stranded antiparallel β sheets that assemble into a propeller-like barrel structure (Díaz et al., 1997). Such a structure is reminiscent of the heterotrimeric G protein β subunit, which interacts with the Ras-like GTPase domain of the α subunit through loops on one side of the barrel. Crystal structures of other small GTPase domains in complex with their effectors demonstrate that there are multiple ways to bind GTPases. Raf, for example, binds to its GTPase mainly through a single β sheet interaction (Figure 2C) (Nassar et al., 1995). Thus, it may emerge that Rab proteins dictate effector binding specificity by utilizing different binding surfaces that select among a diverse set of RBDs.

In summary, Rab GTPases interact specifically with a subset of effector proteins, which in turn could impart specificity to membrane trafficking at the levels of vesicle translocation, docking, and fusion. Rab effectors will likely emerge as a diverse family of proteins that contain varied functional domains suited to their particular tasks and may contain different classes of RBDs. The molecular interactions that underlie the diversity of Rab/effector binding are likely to represent key recognition events in defining the organization of membrane compartments and therefore represent an important area for future study.

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